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14. ABSTRACT
Colorectal cancer is the second leading cause of cancer death in the United States. Approximately 85% of colorectal cancers are CIN+ (Chromosomal instability) and are associated with poor survival. The molecular mechanisms responsible for the CIN phenotype and hence means to target this pattern of genome instability remains poorly defined. I hypothesize that, post-translational modifications (PTM) of the centromeric nucleosome, specifically on the histone variant, CENP-A, will direct centromere activity, and that perturbations of such could lead to aneuploidy and cancer. We proposed to decipher the pathway that leads to CENP-A α-amino methylation and to determine the function it plays in ensuring the fidelity of chromosome segregation. We have shown here that CENP-A is methylated by NRMT1 both *in vitro* and *in vivo*. CENP-A is methylated before it is deposited into the centromere and that methylation persists throughout the cell cycle. We established that CENP-A α-amino tri-methylation required for ensuring high fidelity of chromosome segregation, and hence preventing aneuploidy and cancer. Importantly, we found that loss of CENP-A α-amino tri-methylation trigger a proliferation advantage and cells form bigger colonies in colony formation assay. Suggesting α-amino tri-methylation of CENP-A is an important post-translational modification necessary for maintaining accuracy of chromosome segregation.

15. SUBJECT TERMS
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Functional Characterization of CENP-A Post-Translational Modifications in Chromosome Segregation

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Introduction

Colorectal cancer is the second leading cause of cancer death in the United States[1]. Chromosomal instability (CIN) and microsatellite instability (MIN) are two major molecular hallmarks of colorectal cancer[2,3]. 85% of colorectal cancers are CIN+ and are associated with poor survival[3]. The molecular mechanisms responsible for the CIN phenotype and hence means to target this pattern of genome instability in colorectal tumors remains poorly defined. I hypothesize that, similar to the nucleosomes of general chromatin, post-translational (PTM) modifications of the centromeric nucleosome, specifically on the histone variant, CENP-A, will direct centromere activity, and that perturbations of such could lead to aneuploidy and cancer. However, the identification of CENP-A PTMs has lagged behind PTMs of other histones because of the lack of a good purification strategy. Using a novel purification strategy we have identified three PTMs on the CENP-A tail, α-amino tri-methylation of initial Glycine, and phosphorylations at S16 and S18. Previously, another phosphorylation was reported at Serine S7[4]. In our original application, we proposed to decipher the pathway that leads to CENP-A α-amino methylation (CENP-A α-N-me3) and to determine the function it plays in ensuring the fidelity of chromosome segregation. We also proposed to determine how its abrogation may cause carcinogenesis and ask whether targeting this PTM is a viable strategy to target colorectal cancer cells. Overexpression of CENP-A in colorectal cancer leads to its mislocalization to chromosome arms resulting in aneuploidy. Until now the only way to inhibit CENP-A function was through shRNA knockdown. However, understanding CENP-A amino terminal tail modification will provide enzymatic and therefore potentially druggable targets to inhibit this pathway.

We have shown here that CENP-A is methylated by NRMT1 both *in vitro* and *in vivo*. CENP-A is methylated before it is deposited into the centromere and that the methylation persists throughout the cell cycle. However, we found an increase in CENP-A methylation during prophase of the cell cycle, an observation that merits further studies. We established that CENP-A α-amino tri-methylation is required for ensuring high fidelity of chromosome segregation, and hence preventing aneuploidy and cancer. Importantly, we found that loss of CENP-A α-amino tri-methylation in colorectal cancer cells results in bigger colonies in colony formation assay suggesting that this lesion triggers a proliferative advantage. Our new data support the hypothesis that α-amino tri-methylation of CENP-A is an important post-translational modification necessary for maintaining accuracy of chromosome segregation.

Body

In this section we discuss our results we have accomplished during the first year of this DOD award. Then we will discuss the importance of our finding in discussion section, and finally we will explain major materials and methods we used.

Results:

NRMT Methylates CENP-A

As part of the original proposal, we raised an antibody against amino-terminally trimethylated CENP-A (me3-CENP-A), in order to readily assess CENP-A methylation *in vivo.* We used a peptide blocking experiment to reveal that the antibody is specific. In

this experiment, only the antibody blocked with me3-CENP-A peptide showed loss of centromeric staining indicating it is specific to methylated CENP-A (included in the proposal) (Fig.1A). We also depleted CENP-A using shRNA and immunostained for me3-CENP-A. The results show that CENP-A knockdown completely abolished centromeric staining (Fig.1B). Both these results indicating that the newly raised antibody recognize methylated CENP-A.

Our previous results show that CENP-A N-terminal methionine is cleaved *in vivo* (Fig.1C)[5]. So far, four post-translational modifications on CENP-A have been discovered, alpha-N-methylation of Glycine 1, and phosphorylations at Serine S7, S16 and S18[4,5]. The majority of CENP-A is tri-methylated at the glycine alpha-amino group after methionine removal[5] (Fig.1C). There are two mammalian enzymes that methylate proteins at their alpha amino terminal, NRMT1 and 2. NRMT1 tri-methylates proteins where as NRMT2 is a mono-methylase[6,7]. We purified His-tagged human NRMT1 from bacteria and did an *in vitro* methylation assay. To obtain CENP-A N-terminus devoid of methionine, we expressed a CENP-A fusion protein where a 6XHis tag followed by Factor X protease cleavage site and amino acids 2-10 of CENP-A initial 10 amino acids were fused to the N-terminus of GFP (Fig1.D). The expressed fusion was then purified with nickel beads (Fig.1D), cleaved with Factor X, and negatively selected with nickel beads (Fig.1D). This exposes the glycine 1, which is the first amino acid of CENP-A N-terminus after methionine. This exposed CENP-A amino terminal fusion protein was used as substrate in an *in vitro* NRMT methylation analysis. The proteins then run on a gel and western blotted for GFP and me3-CENP-A antibody (Fig.1E, also shown in the proposal). The me3-CENP-A antibody recognized a band only in reactions having NRMT, clearly showing NRMT1 methylates CENP-A and it again shows that the antibody is specific to methylated CENP-A.

We depleted NRMT1 using shRNA in HeLa cells stably expressing a CENP-A-LAP (localization and purification tag containing GFP) construct. After NRMT1 shRNA expression, NRMT1 was reduced to less than 10% of control shRNA treated cells (Fig.1F). CENP-A methylation was completely lost after NRMT depletion, as shown by western blotting (Fig.1F) and immunofluorescence (Fig.1G,H), without affecting total CENP-A level. This demonstrates that NRMT1 methylates CENP-A both *in vitro* and *in vivo*.

CENP-A methylation is independent of its phosphorylation

We made different mutations at the N-terminal end of CENP-A that were designed to render CENP-A resistant to NRMT1 methyltransferase. Wild type sequence 'GPRRRRS—' was changed to AGPRRRS (MT1), GKRRRS (MT2), GPQQRS (MT3), GPQRRRS (MT4), GPQQRRRS (MT5) or GPQRRS (MT6) (Fig.2A). To verify that these mutations eliminated the ability of NRMT1 to methylate CENP-A, these mutants were expressed as fusion protein as described in fig.1D and 2A. After purifying the proteins with Nickel beads, the 6His tag was removed by Factor X cleavage. The purified and cleaved proteins were used as substrate for our NRMT filter-binding assay. One microgram of substrate was mixed with NRMT1 and radiolabelled SAM was used as the methyl donor. The results show that,all other mutants, except mutants 4 and 5, were not

methylated by NRMT1 (Fig.3B). MT4 and 5 are partially methylated (Fig.3B). Since serine 7 is close to the mutation site, we asked whether these mutations affect serine 7 phosphorylation by Aurora B. We did an *in vitro* kinase assay with recombinant Aurora B (kindly provided by Prof. Stukenberg). Aurora B phosphorylates all mutants except MT3 and MT6 (Fig.2C). However, mutants MT4 and MT5 are better substrate for Aurora B. Hence we selected MT1, 2 and 3 for our further studies. Mutant 3 is neither methylated nor phosphorylated at S7 because this mutation abrogates Aurora B consensus site.

Figure 1

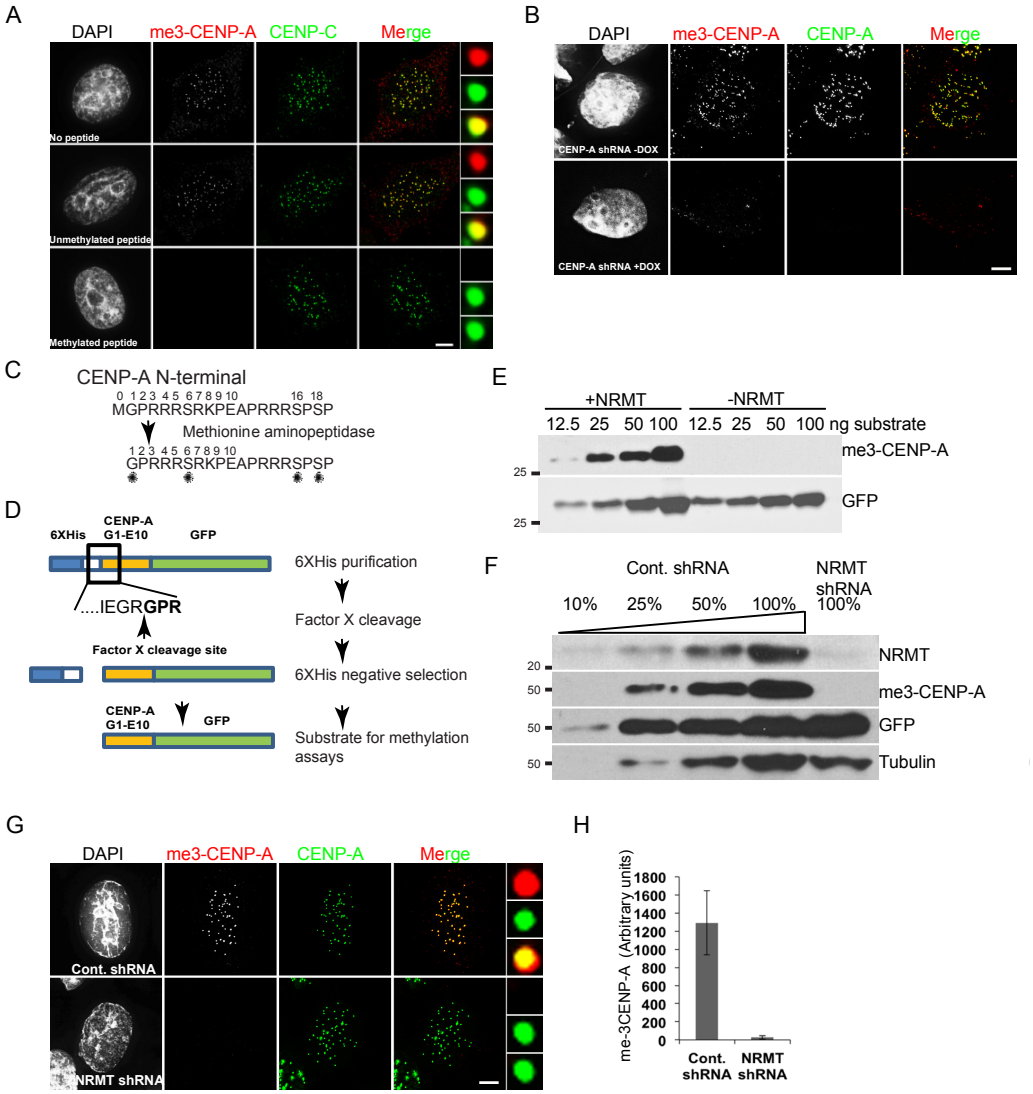

Figure 1. NRMT1 methylates CENP-A *in vitro* and *in vivo* (A). Peptide blocking experiment showing anti me3-CENP-A antibody specifically blocked by methylated peptides. (B). CENP-A knockdown followed by Immunofluorescence analysis in HEK cells using me3-CENP-A antibody, (C). Cartoon of the CENP-A N-terminal tail amino acids showing methionine aminopeptidase cleavage and removal of methionine. Dotes represent post-translationally modified amino acids, (D) Purification scheme for CENP-A N-terminal tail 10 amino acids without beginning methionine. Proteins expressed in bacteria purified with Ni-NTA columns and then cleaved with Factor X this expose glycine for modification. 6XHis tag along with Factor X recognition site was removed by negative selection with Ni-NTA column and the eluate used as substrate for methylation reactions, (E) *In vitro* methylation of CENP-A N-terminal tail. Purified CENP-A tail treated with NRMT and western blotted for me3-CENP-A antibody showing NRMT1 methylates CENP-A, (F). Knockdown of NRMT1 followed by western blotting in HeLa cells showing loss of CENP-A α-N trimethylation, (G). Immunofluorescence analysis in NRMT1 depleted HeLa cells using me3-CENP-A antibody showing loss of CENP-A methylation, (F). Quantitation of methylated CENP-A in NRMT1 depleted cells cells.

We made CENP-A methylation mutants fused with a LAP tag in the pcDNA3-FRT-LAP mammalian expression destination vector. In addition, we also made single and combinatorial alanine substitution mutations of S7, S16, and S18. Stable cell lines were established in HeLa T-Rex Flp-IN cell lines (now on HeLa T-Rex). Lysates from these cells were used for western blotting using anti phospho-S7 antibody (Fig.2D), me3-CENP-A (Fig.2E) and anti phospho-S16;18 antibodies (Fig.2F). As expected, MT3 and S7A mutants were not phosphorylated at S7 (Fig.2D). Immunoblot of non-phosphorylated

Figure 2

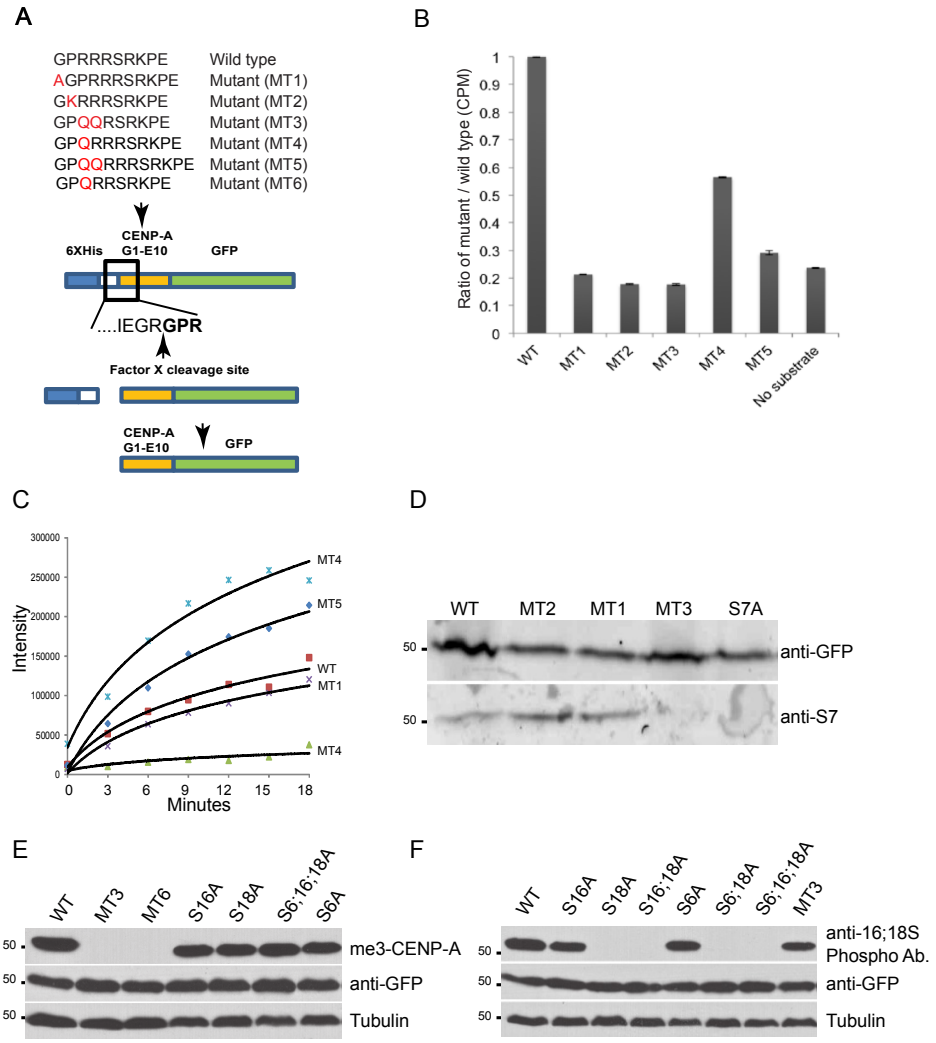

Figure 2. CENP-A methylation is independent of other known CENP-A tail post-translational modifications (A). Schematic of the 2-10 amino acids of the CENP-A tail fused with GFP on the C-terminus and 6XHis on the N-terminus. Factor X cleavage exposes the N-terminal glycine for modification by NRMT. Different mutation made at the N-terminal also shown, (B). *In vitro* NRMT methylation assay. The factor X cleaved CENP-A tail was treated with NRMT in the presence of ^{3}H-S-adenosyl-methionine. A filter-binding assay was used to determine the incorporation of radioactive methyl groups. Mutants 1 to 3 were not methylated, (C). Kinase assay using Aurora B enzyme and CENP-A wild type and mutant substrates. Graphs showing amount of phosphorylation at different time points. Mutant 1 phosphorylated similar to wild type where as mutant 3 not phosphorylated. Mutant 4 and 5 were more phosphorylated. (D). CENP-A wild type and mutants were western blotted for S7 phosphorylation. Mutant 3 and S7A were not phosphorylated (E,F). CENP-A S16 and S18 phosphorylations are independent of methylation. CENP-A methylation and phospho mutants were blotted for me3-CENP-A and phospho antibodies respectively. Me3-CENP-A antibody detected all phosphorylation mutants and anti-16;18 phospho antibody detected methylation mutant MT3, suggesting phosphorylations and methylation are independent events.

CENP-A constructs using the me3-CENP-A specific antibody showed that methylation did not require phosphorylation of the CENP-A tail. (Fig.2E). Similarly, the S16;18 phospho-antibody detected methylation mutants showing these phosphorylation did not require methylation (Fig.2F). Similarly, CENP-A's phosphorylations are not affected by loss of CENP-A methylation. The results show that CENP-A is methylated irrespective of other modifications, indicating all these events are independent (Fig.4).

Figure 3

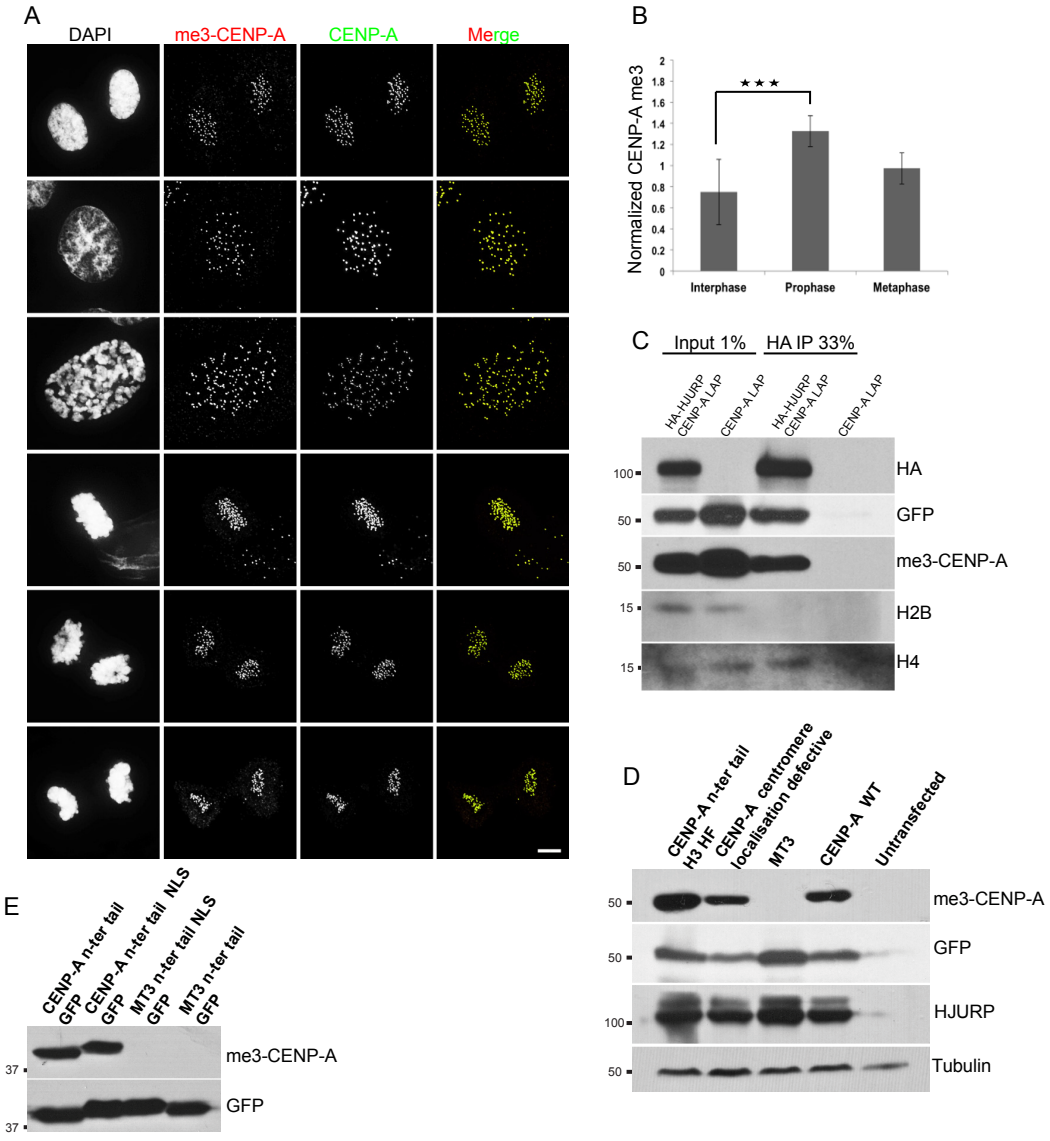

Figure 3. CENP-A methyalted throughout the cell cycle and the centromere localization not required for its methylation (A). Cell cycle distribution of methylated CENP-A at the centromeres, (B). Quantitation of the methylated CENP-A at the centromeres showing a significant increase in methylated CENP-A during prophase. (C). Pre-nucleosomal CENP-A is methylated. CENP-A (GFP) was co-transfected with HA-HJURP and IP was done with anti-HA antibody in chromatin free extract and western blotted for me3-CENP-A, The lack of H2B in the IP fraction and presence of H4 indicating the pulled down CENP-A is pre-nucleosomal. (D). Centromere localization is not required for CENP-A methylation. CENP-A tail fused with H3.1 histone fold (H3HF) and a CENP-A centromere localization defective mutant methylated *in vivo*, (E). Methylation of CENP-A is independent of its histone fold. The cells were transfected with CENP-A tail fused with GFP and western blotted for me3-CENP-A.

CENP-A is methylated throughout the cell cycle and centromere localization is not required for its methylation

To determine the cell cycle phase at which CENP-A is methylated we stained randomly cycling U2OS cells with me3-CENP-A antibody. We found that CENP-A was methylated throughout the cell cycle. In order to identify changes in the amount of methylated CENP-A at centromeres we compared the ratio of methylated to total CENP-A, to take into account any changes in total CENP-A levels. Using this measure we observed an increase in me3-CENP-A during prophase (Fig.3A,B). We confirmed the prophase increase in CENP-A amino terminal methylation in the colorectal cancer cell line, HCT116. Given the timing of the CENP-A methylation increase, we hypothesize that increased methylation may be required for accurate segregation of chromosomes.

Figure 4

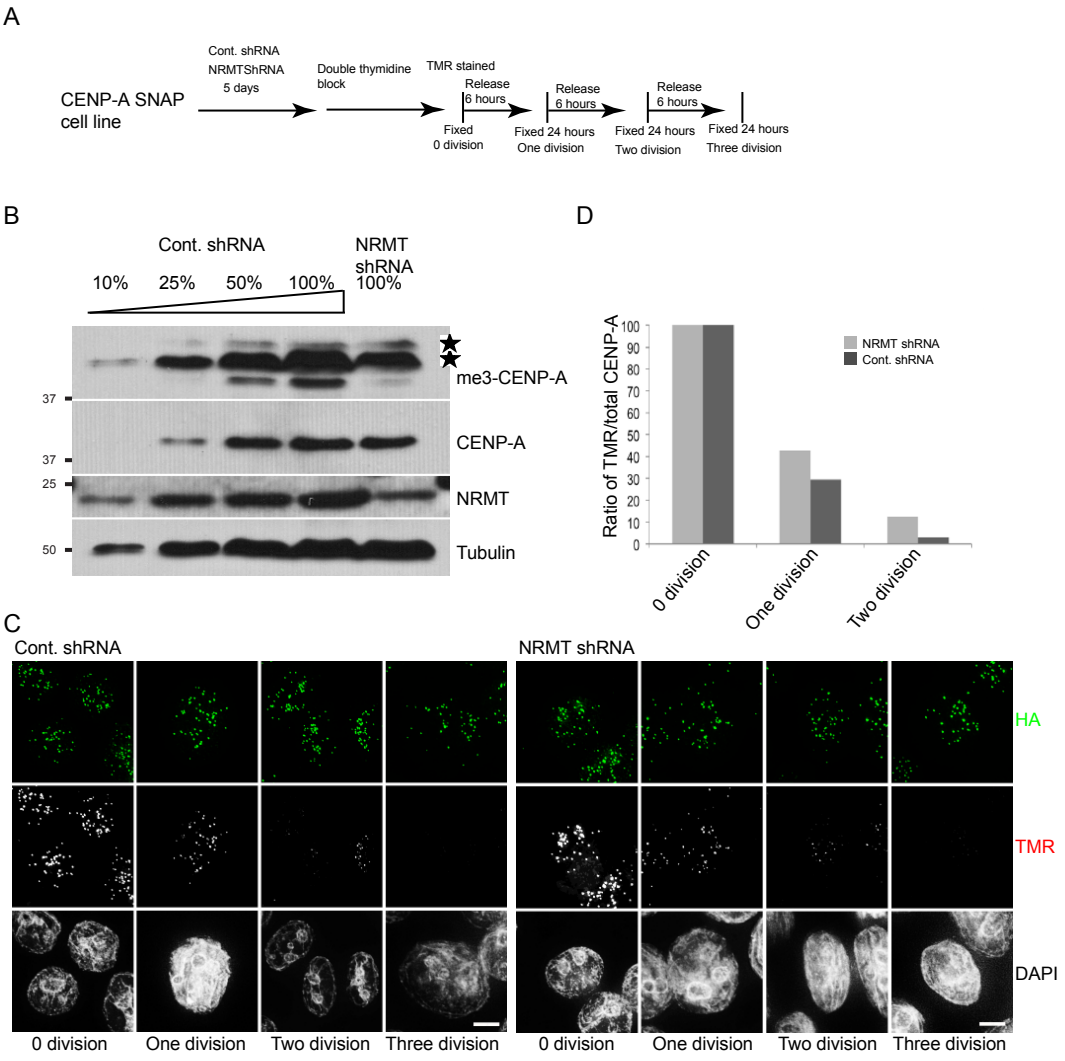

Figure 4. CENP-A methylation not required for its stability (A). Schematic diagram of the experiment. CENP-A SNAP cell lines were stably integrated with NRMT shRNA. Cells were TMR stained after double thymidine block and released to different time points (B). Western blots showing NRMT depletion and loss of CENP-A methylation. Stars indicate nonspecific bands, (C). Immunofluorescence analysis showing total CENP-A (HA) and TMR stained CENP-A after consecutive cell division, (E). Quantitation of the immunofluorescence analysis, ratio of the TMR/total CENP-A showed. CENP-A's stability was not compromised after NRMT1 knockdown.

Additional experiments will be conducted in year 2 and 3 of this proposal to deduce the relevance of this increased CENP-A methylation during the beginning of mitosis.

To determine whether CENP-A is methylated before it is deposited at the centromere, we compared pre-nucleosomal and nucleosomal fractions from cells co-transfected CENP-A-LAP and HA tagged HJURP. The chromatin free cell lysate was prepared using hypotonic lysis and then HJURP was pulled down using HA antibody. Pre-nucleosomal CENP-A forms a complex with its chaperone HJURP and histone H4. The IP shows an absence of histone H2B and presence of histone H4, clearly demonstrating we pulled down pre-nucleosomal CENP-A (Fig.3C). The western blot using me3-CENP-A antibody shows pre-nucleosomal CENP-A is methylated (Fig.3C). We transfected a mutant CENP-A that does not interact with HJURP and hence is not localized at the centromere and a construct encoding the N-terminal tail of CENP-A fused with H3 histone fold domain (H3HF). Both these mutants were methylated (Fig.3D). Moreover when a construct expressing the CENP-A N-terminal tail fused with GFP (CENP-A N-terminal GFP) was transfected into cells it was also methylated. Indicating centromere localization of CENP-A is not required for its methylation (Fig.3E).

Methylation not required for CENP-A nucleosome stability

To determine whether CENP-A methylation is required for nucleosome stability, we conducted an in vivo pulse-chase experiment using SNAP-tag as described previously[8]. The SNAP tag is a modified variant of the suicide enzyme O^6-alkylguanine-DNA alkyltransferase that irreversibly modifies (and inactivate) itself through acceptance of the cell-permeable guanine derivative O^6-benzylguanine (BG, non-fluorescent; or TMR-star, fluorescent). This system allows labeling of SNAP fusion proteins *in vivo*. We used pulse labeling with this methodology to determining CENP-A turnover specifically at centromeres with or without NRMT1 knockdown. We established cell lines stably expressing both centromere-localized CENP-A–SNAP and either control shRNA or NRMT1 shRNA plasmids. The cells were synchronized at the G1–S boundary by double thymidine block. After second thymidine block CENP-A was pulse-labeled with TMR-*Star* and were chased for up to two cell cycles (Fig.4A). The western blot shows approximately 90% of NRMT1 was depleted and CENP-A methylation was reduced significantly (Fig.4B). After first cell division, the centromere TMR-star labeled CENP-A-SNAP was reduced to 40% in NRMT1 knockdown samples and 35% in control shRNA transduced cells. After a second cycle of DNA replication, the previously labeled centromere-bound CENP-A–SNAP was reduced to less than 10% and 5% of its initial level in knockdown and control samples respectively. These reductions are consistent with dilution of CENP-A nucleosomes during DNA replication. Since we observed no difference in the reduction of CENP-A between control and NRMT shRNA treated cells, these preliminary results suggest CENP-A's stability is not compromised the lack of CENP-A methylation.

CENP-A depletion causes multipolar spindle formation in a p53 dependent manner

CENP-A histones epigenetically determine the centromere, which is where kinetochore forms during mitosis and accurately segregate chromosomes. Overexpression of CENP-A leads to its mislocalisation and missegregation of chromosomes[9]. Similarly loss of CENP-A also causes missegregation of chromosomes by improper kinetochore

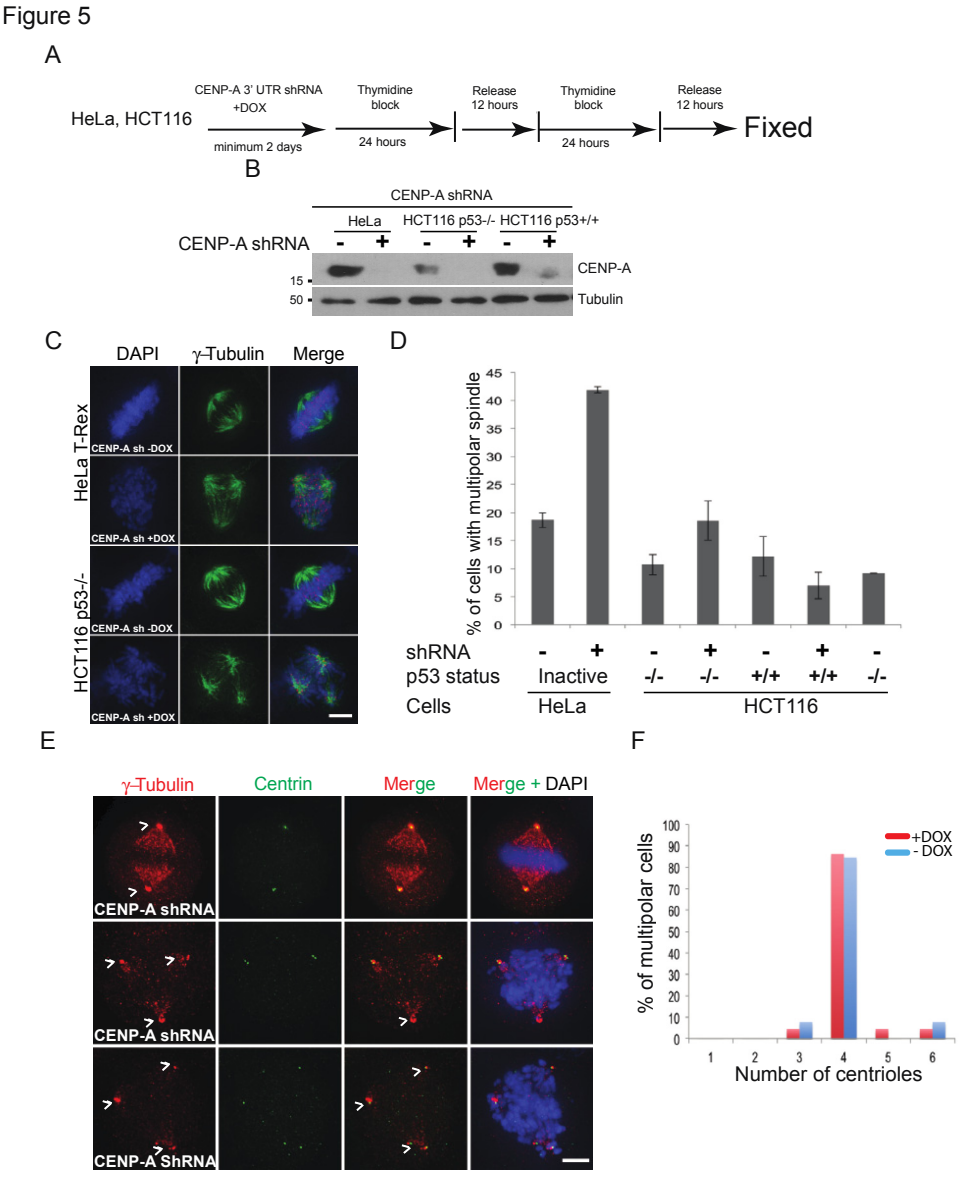

Figure 5. CENP-A knockdown leads to multipolar spindle in p53 inactive cells (A). Schematic diagram of the experiment. CENP-A shRNA was induced for two days before starting the double thymidine block and release. Cells were fixed 12 hours after second release, (B). Western blots show depletion of of CENP-A after induction of shRNA using doxycycline (DOX), (C) CENP-A knockdown causes multipolar spindle. Cells were stained for α□tubulin after CENP-A knockdown, (D). Percentages of the multipolar cells. HeLa and HCT116 p53-/- cells show higher percentages of multipolar cells, (E). Examples of bipolar and multipolar cells stained for γ□tubulin and centriole marker centrin showing centrosomes without centriole. The arrows in the first column indicate centrosomes and in the third column the centrosomes with no centriole or one centriole (F). Number of centrioles in multipolar cells. The majority of cells have only four centrioles both in control and CENP-A knockdown samples.

Figure 6

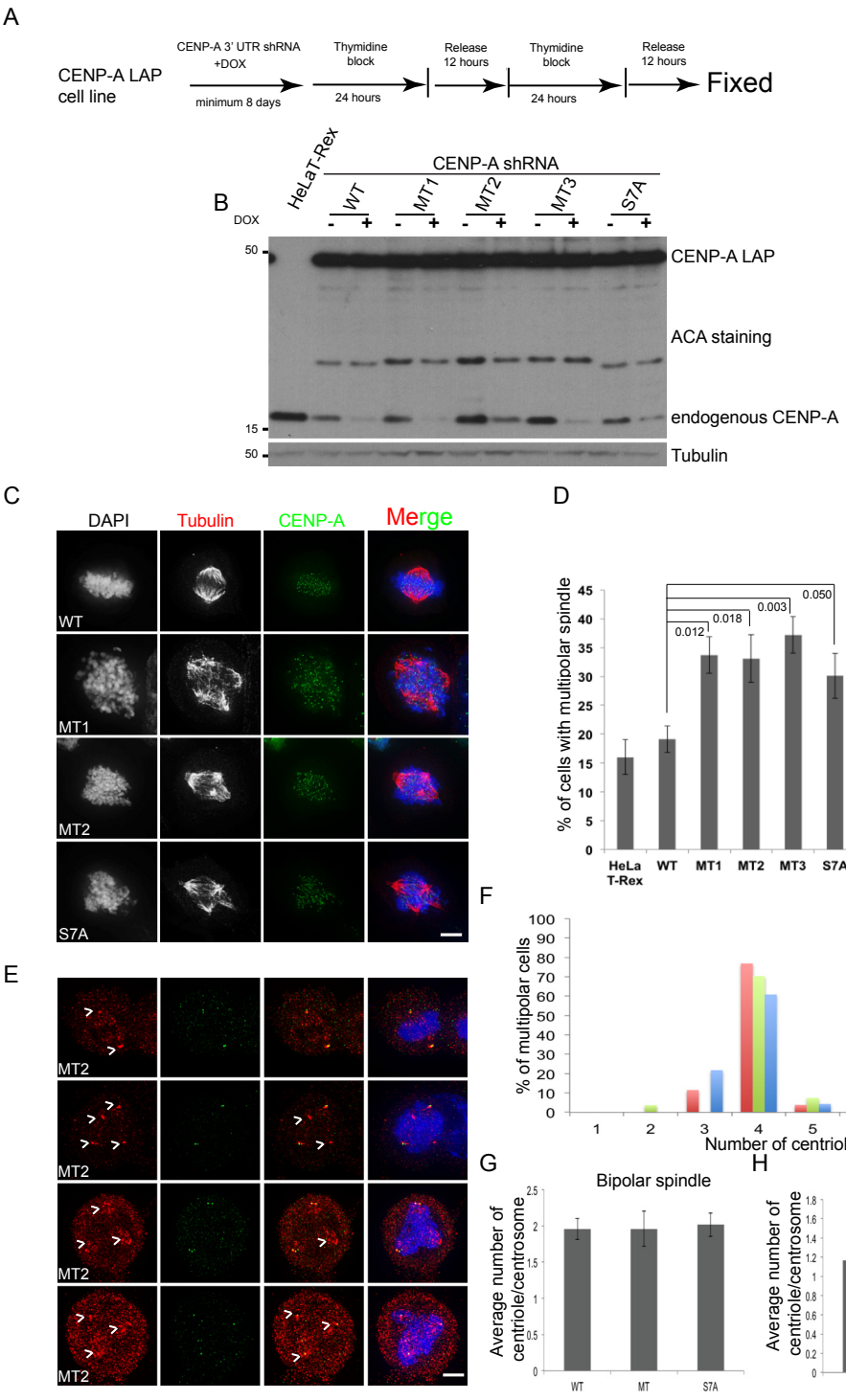

Figure 6. Loss of CENP-A methylation causes multipolar spindle in HeLa cells (A). Schematic diagram of the experiment. CENP-A shRNA was induced for 8 days before starting the double thymidine block and release. Cells were fixed 12 hours after second release, (B). Western blots of the CENP-A-LAP wild type and methylation mutant stable cell lines stably integrated DOX inducible CENP-A 3'UTR shRNA before and after DOX induction. The membrane was probed with ACA showing endogenous CENP-A reduced after knockdown, (C). Methylation deficient mutants (MT 1,2 and 3) of CENP-A causes multipolar spindle (D). Quantitation of the multipolar cells. Multipolar cells were significantly different from both parental cells and wild type replaced cells. S7A mutant also causing multipolar

spindle, (E). Examples of γ-tubulin stained multipolar cells. Majority of cells has one or more centrosome without centrioles. The arrows in the first column indicate centrosomes and in the third column depicting centrosome with one or no centriole, (F). Number of centrioles per multipolar cells are shown. Majority of cells have only four centrioles. Indicating lack of centriole duplication as a mechanism to form multipolar cells, (F,G). Average number of centrioles per centrosome in multipolar and bipolar cells. Multipolar cells have less centriole per centrosome.

formation[10]. In the presence of p53, loss of CENP-A induces senescence in immortalized cells[10]. To determine the phenotype of CENP-A loss in tumor cells, we depleted CENP-A in p53 wild-type and null isogenic HCT 116 colorectal cancer cells[11] as well as in a HeLa cell line using shRNA against the CENP-A 3' UTR. P53 is inactivated in HeLa cells due to expression of the HPV E6 protein. We found CENP-A levels were significantly reduced after shRNA induction by addition of doxycycline (Fig.5A,B). Phenotypically, we found that CENP-A depletion caused a significant increase in the number of cells with multipolar spindles in the HeLa T-Rex line (Fig.5C,D). In HCT116 cells, depletion of CENP-A caused more cells to posses multipolar spindles in p53 null cells but not in p53 wild-type cells (Fig.5D). Both these results show that depletion of CENP-A causes multipolar spindle formation in p53 inactive or null cancer cells. We also checked whether the multipolar spindles were formed because of centriole duplication or centrosome splitting (Fig.5E,F). Usually failure in cytokinesis causes centriole duplication. We found that the majority of multipolar cells have only four centrioles (Fig.5F). And also majority of multipolar cells showed at least one γ-tubulin stained centrosome like structures without centriole in it. In some cells centrioles split between centrosome having one centriole per centrosome instead of two. All these results indicated that multipolar spindles induced by CENP-A depletion or expression of methylation constrcuts were formed by centrosome splitting, and not by a failure in cytokinesis or centrosome reduplication.

We determined whether CENP-A depletion causes any increase in lagging chromosome during telophase. As we expected, there was increased number of lagging chromosomes in both p53-/- and p53+/+ isogenic HCT116 cells when CENP-A was depleted. p53+/+ cells showed dramatic increase in lagging chromosomes upon CENP-A depletion. In p53-/- cells, the overall percentage of cells showing lagging chromosomes was initially high and it was shifted upward after CENP-A knockdown (Fig.7E,F). In p53-/- cells showed a significant increase in multipolar spindle formation; however, p53+/+ cells did not, perhaps dues to p53 dependent mechanism known to suppress multipolar spindle formation[12] (Fig.7C-H). All these show that CENP-A is essential for maintaining proper chromosome segregation and bipolar spindle.

CENP-A methylation is required for bipolar spindle formation and error free chromosome segregation

To dissect the function of CENP-A methylation we made CENP-A methylation mutant stable cell lines in HeLa T-Rex (Fig.6) and HCT116 p53-/- and p53+/+ cells (Fig.7). We have made stable cell lines in HCT116 cells by viral integration and the cells expressing the construct were selected. For HeLa T-Rex, we have used the Flp-In method to generate stable cell lines. We then stably integrated doxycycline inducible 3'UTR targeting CENP-A shRNA using lentivirus. Because the shRNA targets the 3'UTR, it selectively depletes endogenous CENP-A, but not our exogenous mutants. Eight days after starting doxycycline-mediated induction of shRNA, the cells were plated on coverslips and we did a double thymidine block and release experiment. 12 hours after

Figure 7

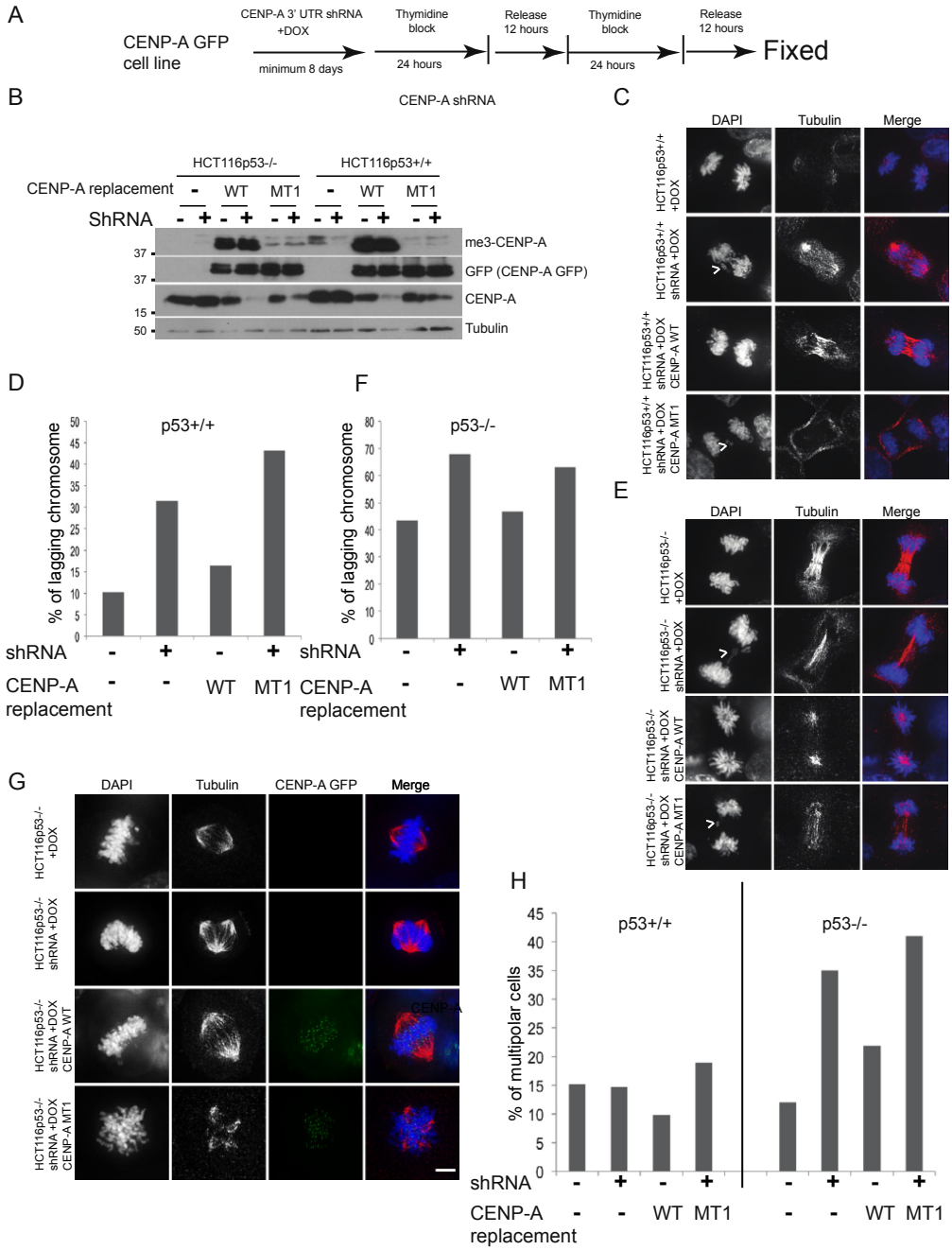

Figure 7. Loss of CENP-A methylation causes lagging chromosomes in p53 +/+ colorectal cancer cells and multipolar cells in p53-/- colorectal cells (A). A schematic diagram of the experiment, (B). Western blots of the knockdown and replacement experiments. Endogenous CENP-A level reduced in the replaced cells and was further reduced in knockdown cells, (C) Knockdown and replacement experiment of CENP-A methylation mutant. Figure showing lagging chromosomes (arrows) in CENP-A knockdown HCT116 p53+/+ cells and was rescued by wild type CENP-A but not mutant (MT1), (D). Graph showing the percentage of cells having lagging chromosomes (arrows) in HCT116 p53+/+ cells in knockdown and replacement experiment of CENP-A methylation mutant, (E). Cells showing lagging chromosomes in CENP-A knockdown HCT116 p53-/- cells and was rescued by wild type CENP-A but not mutant (MT1), (F). Graph showing the percentage of cells having lagging chromosomes, (G). Cells showing multipolar spindle after CENP-A knockdown replacent with methylation mutant (MT1) in HCT116 p53-/-, (H). Percentage of multipolar cells in p53-/- and p53+/+ HCT116 colorectal cancer cells. In p53-/- cells the CENP-A knockdown leads to increased multipolar cells and was partially rescued by wild type CENP-A but not methylation mutant (MT1).

the second release cells were fixed and stained for tubulin and DAPI (Fig.6A,7A). At the same time we also harvested cells for western blotting and the western blot showed a significant depletion of endogenous CENP-A (Fig.6B,7B). We found that the multipolar spindle formation induced by CENP-A depletion (Fig.5C,D) was rescued by CENP-A wild type replacement, but not by methylation mutants and S7A mutant (Fig.6C,D). Interestingly, mutant 3, which is neither alpha-N-methylated nor phosphorylated at S7, showed the highest percentage of multipolar cells. Showing a slight additive effect (Fig.6C,D). Similar to CENP-A depleted cells, the mutant replaced cells also showed centrosome splitting, where two daughter centrioles are found in different poles (Fig.6E,F,G). The majority of multipolar cells have the expected four centrioles, with some centrosomes containing only one centriole or none (Fig.6E).

Similar to the HeLa cells HCT116 p53-/- cells, methylation mutant cells showed increased number of multipolar cells compared to the CENP-A wild-type rescue (Fig.7G,H). However, the percentage of p53+/+ HCT116 cells with multipolar spindles was similar in both CENP-A wild type and mutant cells (Fig.7G,H). Compared to our previous experiment, where we depleted CENP-A only for four days, here we found an increase in multipolar spindle (Fig.5D,6H). Indicating a longer knockdown cause more spindle defects in HCT116p53-/- cells.

We also analyzed chromosome segregation defects in these cells. Any defect in chromosome segregation during telophase was counted. We found a significantly higher percentage of cells showing lagging chromosomes when we depleted CENP-A and replaced with methylation mutant CENP-A in p53+/+ HCT116 relative to rescued with wild type CENP-A (Fig. 7C,D). In p53-/- HCT116 cells, though the overall percentage of lagging chromosomes was initially higher than p53+/+ cells, the depletion and replacement with the methylation resistant CENP-A mutant showed an increase in lagging chromosomes compared to the wild type CENP-A rescue (Fig.7 E,F). Therefore we conclude that CENP-A amino-terminal methylation is required for proper chromosome segregation and its loss leads to aneuploidy in colorectal cancer cells.

Loss CENP-A methylation causes uncontrolled proliferation

To determine the role of CENP-A in cell viability and proliferation, we performed a colony formation assay using the HCT116 wild type and p53 null cells where we depleted CENP-A. We have used the double stable cell line of CENP-A 3'UTR shRNA and either CENP-A wild type or CENP-A methylation mutant 1 for our knockdown replacement experiment. The CENP-A methylation mutant cell line formed bigger colonies in p53-/- cells when compared to wild type cells after 8 days of CENP-A depletion. We also found significantly bigger colonies in p53+/+ cells. These data suggest that, in the absence of p53, the loss/reduction of CENP-A methylation provides proliferative advantage and thus promote tumorigenesis. Whereas, in the presence of p53, the chromosome segregation defects suppressed by p53 but eventually leads to bigger tumors.

Discussion:

The post-translational methylation of alpha-amino groups was first discovered over 30 years ago[3]. However, its biological function remains obscure except in the case RCC1 (Ran guanine nucleotide-exchange factor) where it is required for its localization

into chromosomes[13]. Here we identified a novel function of alpha-N-methylation. We found that alpha-N-methylation of CENP-A is required for maintaining genomic stability and its loss leads to aneuploidy.

We have demonstrated CENP-A is methylated throughout the cell cycle and methylated CENP-A increases during prophase. CENP-A is methylated prior to its deposition. However, we cannot rule out the possibility that a fraction of CENP-A is methylated after its deposition at the centromere. The increase in methylated CENP-A during prophase may suggest such a possibility. The increased methylation prior to metaphase is consistent with a role of methylation in chromosome segregation. Although we do not know the direct consequences of CENP-A methylation as of yet, the next two years of support form the DOD will allow us to determine the molecular interaction that underlie this effect.

Figure 8

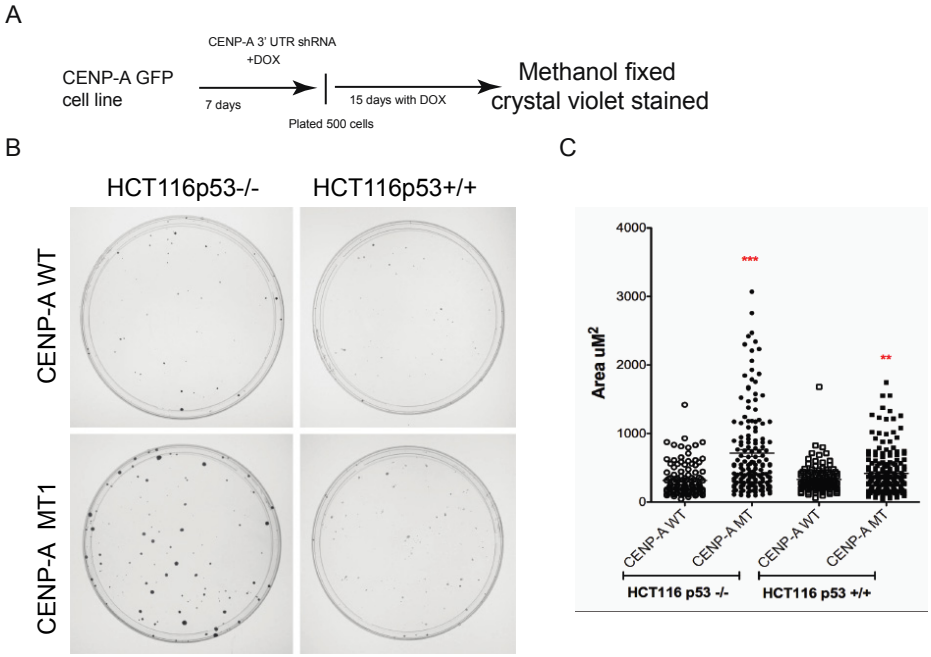

Figure 8. Loss of CENP-A methylation causes increased cell proliferation and survival in a p53 dependent manner (A). A schematic diagram of the experiment. CENP-A-GFP wild type or mutant constructs were stably integrated along with DOX inducible CENP-A 3'UTR shRNA in p53+/+ and p53-/- HCT116 cells lines. Dox was added to the double stable cells for seven days and 500 cells from each experiment was plated for the colony formation assay. The cells were grown under continued presence of DOX. After 15 days cells were methanol fixed and stained with crystal violet, (B). Representative pictures of the colony formation assay. (C). Quantitation of the area of the colonies. Area of the colonies significantly different between CENP-A wild type and mutant both in p53-/- (<0.0001) and p53+/+ (0.0091) HCT116 cells.

Reduction in CENP-A level increases the propensity of cells to form multipolar spindle pole and lagging chromosome in p53 null colorectal cancer cell HCT116. Whereas, in isogenic p53+/+ cells there is no significant increase in multipolar spindle formation after CENP-A knockdown; however there is a dramatic increase in lagging chromosome. Moreover, we also found increased multipolar cells in p53 inactive HeLa cells, suggesting that reduction of CENP-A causes aneuploidy either by multipolar spindle formation or by forming lagging chromosomes or both. It has been previously

shown that NRMT1 depletion cause multipolar spindle in HeLa cells[6]. We wondered whether this effect is through a hypomorphic CENP-A that is methylated by NRMT1[3]. In our shRNA depletion replacement experiment, cells rescued with wild type CENP-A had similar percentage of multipolar cells as that of parental cell lines both in colorectal cancer cell HCT116 p53-/- and HeLa. But rescue with the methylation mutant CENP-A showed significantly increased multipolar spindle in p53-/- HCT116 and p53 inactive HeLa cells, suggesting CENP-A methylation mutants are hypomorphic.

We also analyzed chromosome missegregation defects and found that there is an increase in lagging chromosomes compared to wild type irrespective of p53 status. The lagging chromosomes occur when kinetochores form improper microtubule attachments. We are currently looking for changes in centromere and kinetochore associated proteins in these cells to determine any defects in their localization. In normal cells it may be possible that loss of CENP-A methylation drive chromosome missegregation and when p53 is mutated it induce tumorigenesis or vice versa. It is important to look the methylation status of CENP-A in colorectal cancer tissues to see whether methylated CENP-A is reduced during tumorigenesis and tumor progression.

We also found further evidence that CENP-A methylation has a protective effect in tumorigenesis. In the colony formation assay when endogenous CENP-A is replaced with wild type or methylation mutant by depletion and replacement, the mutants formed larger colonies. This effect was more pronounced in p53-/- HCT116 cells. All these results suggest a role of CENP-A methylation in normal cell division and proliferation. Our model suggest that overexpressed CENP-A, which is very common in colorectal cancer may not be fully methylated and that may cause chromosome instability and multipolar spindles. This may lead to more aggressive tumors. We have made exciting progress in the proposed study and continuation of this study is necessary to fully understand the function of alpha-amino group methylation of CENP-A. In the next year we will focus on to elucidate the mechanism of the chromosome segregation defects in the methylation mutants. We will look alteration in the localization of the centromere and kinetochore associated complexes in methylation mutants. We will also look at the status of CENP-A methylation in colorectal tumor samples to see its direct association with cancer progression and survival.

Materials and methods:

***In vitro* methylation assays:** 6XHis tagged Human NRMT1 (Gift from Ian Maccara) was purified from E- *coli* and used for the methylation assays. CENP-A initial 2-10 amino acids were purified as a fusion protein. 6XHis followed by Factor X cleavage site and then CENP-A ten N-terminal amino acids starting from second amino acid Glycine fused with GFP's N-terminal. The fusion proteins were expressed from a modified pET15b (Novagen) expression vector in BL21 *E. coli* and purified on Ni-NTA beads and then cleaved using Factor X (Sigma-Aldrich). The cleaved proteins were then negatively selected with Ni-NTA column and the eluate used as substrate for methylation assays. Reactions (50 µl) were performed in MTase buffer (50 mM Tris, 50 mM potassium acetate, pH 8.0), using 3.0 pmol of recombinant NRMT plus 1µg of purified substrates. The reaction was incubated for 2 h at 30 °C after addition of 1 µl of ^{3}H-SAM (0.55 µCi µl^{-1}). Reactions were filtered through nitrocellulose, washed with 50 mM sodium bicarbonate and subjected to scintillation counting. For western blotting

analysis, cold SAM was used instead of ^{3}H-SAM.

shRNA mediated knockdown of NRMT1 and CENP-A: The human and mouse NRMT lentiviral shRNAmir pGIPZ constructs were obtained from Ian Maccara's lab. The targeting sequence of the shRNA against NRMT is AGAGAAGCAATTCTATTCCAAG; and the control sequence is CCCTGCCAGACAGTACCAATTA. To make virus, 2.5×10^6 293LT cells were calcium-phosphate-transfected with 20 µg of the NRMT pGIPZ plasmid, 6 µg of the vesicular stomatitis virus coat protein plasmid (pMD2G), and 15 µg of packaging plasmid (psPAX2)[6]. Viral supernatants were collected after 48 h and infected HeLa T-Rex or HCT116 cells. After 3 days, 2 µg ml^{-1} puromycin was added to select transduced cells. For CENP-A knockdown, doxycycline inducible shRNA targeting 3'UTR on pTRIPZ constructs was used. The viruses were made as described for NRMT1 and infected HeLa T-Rex and HCT116 cells. CENP-A equally distributes between daughter chromosomes during DNA replication. It takes at least four cell divisions to reduce CENP-A below 10% level. For CENP-A knockdown, shRNA was induced two days before starting double thymidine block and release so that the cells undergo approximately four cell divisions to get effective knockdown.

For knockdown and replacement experiments in HCT116 cells, CENP-A wild type and mutants were cloned into pBABE retrovirus vector by cold fusion. CENP-A fused with GFP on the C-terminus. The viruses were packaged by transfecting into 293 GP cells along with VSVG plasmid. The viruses were collected after 3 days. For making double stable cell lines, the cells were co-infected with CENP-A retrovirus and CENP-A 3'UTR shRNA lentivirus. The double stable cells were selected using 6 µg ml^{-1} Blasticidin and 2 µg ml^{-1} puromycin. CENP-A was also cloned into modified pcDNA3/FRT-LAP destination vector kindly provided by Stukenberg lab. HeLa T-Rex cells were transfected with CENP-A wild type or mutants along with FLP-recombinase. The stable cells were selected with hygromycin. The stables cells were then infected with doxycycline inducible CENP-A shRNA virus to establish double stable cell lines. For knockdown replacement experiments shRNA was induced 8 days before starting double thymidine block and release to get effective endogenous CENP-A depletion.

Peptide blocking: Cells were pre-extracted for 3 minutes using 0.1% triton followed by fixation in 4% formaldehyde for 10 minutes. The cells were then blocked in 1XPBS containing 2% BSA, 2% fetal calf serum and 0.1% triton for one hour. Meanwhile me3-CENP-A antibody was pre-incubated with methylated or unmethylated CENP-A N-terminal peptide at a concentration of 10X peptide to 1X antibody for 30 minutes at room temperature. The antibody then centrifuged and the supernatant used for staining as described in immunofluorescence section.

Immunofluorescence: Cells were either pre-extracted with 0.1% triton in 1X phembuffer (60mM PIPES, 25mM HEPES, 10mM MgCl2) for 3 minutes and then fixed in 2% formaldehyde for 10 minutes or fixed in 2% formaldehyde for 10 minutes and then permeabilized in 0.1% triton for 5 minutes. The cells were then blocked in 2% BSA and 2% fetal calf serum with 0.1% triton in 1XPBS. It was incubated with primary antibodies one hour and then secondary antibody was added for one hour. All washes between

each step were done in 1XPBS + 0.1% triton. DNA was stained with DAPI and were mounted in prolong. Cells were examined and images were acquired using a Delta Vision optical sectioning deconvolution instrument (Applied Precision) using a X100 oil-immersion Olympus objective lens connected with Photometrics CoolSNAP HQ^2 camera and Softwrox acquisition software. Images were deconvolved and presented as maximum stacked images. For staining γ-tubulin and centrin, cells were fixed in ice-cold methanol on ice for 3 minutes and then proceeded with antigen blocking as described above. The antibodies used for immunofluorescence were: mouse anti-CENP-A (1:1000), Rabbit anti-me3CENP-A (1:200), mouse anti-CENP-A (1:1000), Rabbit anti-CENP-T (1:1000), Rabbit anti-□□-tubulin (1:1000; Sigma), Mouse anti-centrin (1:1000; Millipore), Mouse anti-α-tubulin (1:1000).

Following antibodies were used for IP and western blotting. Rabbit anti-GFP (1:3000), Rabbit anti-H2B (1:1000), Rabbit anti-H4 (1:5000), Mouse anti-HA (1:1000), Mouse anti-tubulin (1:1000).

SNAP tagging and pulse chase experiment: CENP-A–SNAP–3XHA cell lines previously described used in this study[5]. NRMT1 or control shRNA was stably integrated into this cell lines. Cells were then double thymine blocked using 2mM thymidine. During the end of second thymidine block, the cells were labeled with 2 μM TMR-*Star* (Covalys) in complete growth medium for 60 min at 37°C. Labeling was followed by one wash each with PBS, and DMEM and incubated for 30 min, and washed with PBS prior to fixation (0 division). The cells were then released 6 hours before adding 2mM thymidine and cells were fixed after 12 hours after release (1 division). Similarly cells were released and fixed for second and third divisions. Total CENP-A was then stained with anti-HA antibody and counter stained with DAPI.

Key accomplishments:

Following are the key accomplishments of the proposed work

1. Identified NRMT1 methylates CENP-A at its α-amino terminal.
2. CENP-A is methylated throughout the cell cycle.
3. Pre-nucleaosomal CENP-A is methylated.
4. We made several methylation resistant CENP-A mutants.
5. Loss of CENP-A cause aneuploidy by forming multipolar spindle and missegregation of chromosomes.
6. α-amino tri-methylation of CENP-A ensures high fidelity of chromosome segregation. Its loss cause multipolar spindle and lagging chromosomes in p53 null background and lagging chromosomes in the presence of p53. Both these alterations fundamentally cause aneuploidy, a form of genetic alterations prevalent (85%) in colorectal cancer.
7. We also found that methylation mutant CENP-A forms bigger colonies in colony formation assay suggesting role of methylation in controlling proliferation.

We have presented a poster regarding the role of CENP-A α-amino tri-methylation in accurate chromosome segregation and cancer during the international Keystone Cancer Epigenetics conference held in February 2014 (poster attached).

Reportable outcome:

All the eight figures in the results section will form the basis of a manuscript we are planning to write. We have found that CENP-A α-amino tri-methylation is a crucial post-translational modification in maintaining high fidelity of chromosome segregation and any defect in this modification may results in aneuploidy and cancer.

Meeting attended/presented

Poster, Cancer Epigenetics, Keystone symposia, February 4-9, 2014.

N-terminal α-amino trimethylation of centromere histone CENP-A required for maintaining bipolar spindle and regulated cell proliferation. Kizhakke M. Sathyan, Aaron O. Bailey, Tanya Panchenko, Donald F. Hunt, Ben E Black, Daniel R. Foltz. Cancer Epigenetics, Keystone symposia, February 4-9, 2014.

Abstract of the poster:

The deposition of histone H3 variant CENP-A marks centromere in higher eukaryotes, chromosomal loci required for accurate segregation of chromosome. It has been shown a majority of cancers exhibits chromosomal instability, however, the mechanism is unclear. Overexpression of CENP-A and its chaperone HJURP occurs in many cancers and is sufficient to cause chromosome missegregation in cell culture. The posttranslational modification (PTM) of histones resulting in the recruitment of activator and repressive complexes to chromatin is a well established and broadly used method to regulate chromatin activity by the cell. We propose that the PTMs of CENP-A have a major role in preventing chromosomal instability. Despite the identification of nearly 14 PTMs on the histone H3 amino terminus, many of which have significant functional consequences, prior to our work only a single phosphorylation on serine 7 (pSer7) of CENP-A has been described in higher eukaryotes. Recently, we have reported three new PTMs on human CENP-A N-termini using high-resolution MS: trimethylation of Gly1 and phosphorylation of Ser16 and Ser18. The nascent N-terminal residue Gly1 becomes trimethylated on the α-amino group following initial methionine removal. Here we demonstrate that the N-terminal RCC1 methyltransferase (NRMT1) methylates CENP-A both *in vitro* and *in vivo*. Even though it was identified 30 years ago, the function of N-terminal α-amino group methylation is poorly understood. Methylation occurs in the prenucleosomal as well as nucleosomal forms of CENP-A, although nucleosomal CENP-A showed higher methylation. *In vivo* as well as *in vitro* data suggest that the amino terminal tail of CENP-A is sufficient for trimethylation. We also

found significant differences in methylation of centromeric CENP-A during prophase of the cell cycle. The CENP-A methylation resistant mutants show multipolar spindles, multiple centrioles and multinucleated cells indicating a failure of cytokinesis in p53 inactivated HeLa cells. We also found similar results in p53-/- HCT116 cells. The methylation mutant cells formed larger and higher number of colonies indicating uncontrolled growth in p53-/- cells, whereas flatter cells in p53+/+ cells may be due to induction of senescence. We are currently analyzing how the loss of CENP-A α-amino tri-methylation leads to multipolar spindles and uncontrolled cell growth. Supernumerary centrosomes are a common characteristic of cancer cells and a source of chromosome instability. Our work provides a novel link between CENP-A posttranslational modification and the generation of chromosome instability through changes in centrosome number and cell proliferation

Conclusion:

We have made significant progress in elucidating the function of α-amino tri-methylation of CENP-A. We achieved several of the proposed aims. We found that CENP-A is methylated by NRMT1 and this modification persists throughout the cell cycle. We also found that α-amino tri-methylation of CENP-A is critical in orchestrating chromosome segregation and its abrogation may leads to aneuploidy and cancer. Currently, we are analyzing how CENP-A α-amino tri-methylation orchestrating chromosome segregation. We are looking at alterations in proteins associated with the centromere and kinetochore in the CENP-A mutant stable cell lines. We also want to look at the status of CENP-A tri-methylation in colorectal cancer in year 2 and 3 of this proposal. This will tell us the role of α-amino tri-methylation of CENP-A in cancer progression and survival. Further support is necessary to fully understand the function of α-amino tri-methylation of CENP-A and its role in colorectal cancer.

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Appendices:

Abbreviations used

PTM	Post-translational modifications
CIN	Chromosomal instability
MIN	microsatellite instability
NRMT1	N-Terminal RCC1 Methyltransferase 1
NRMT2	N-Terminal RCC1 Methyltransferase 2

shRNA	short hairpin RNA
LAP tag	Localization and Affinity Purification tag
SNAP tag	Modified human O^6-alkylguanine-DNA alkyltransferase
SAM	S-adenosyl methionine
UTR	untranslated region
CENP-A	Centromere protein-A
ACA	Anti-centromere antibodies
α-N-me3	alpha N-terminal amino acid trimethylation